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In vitro Viability and Fertilizing Capacity of Guinea Fowl Spermatozoa

Noboru Fujihara, Hisayoshi Nishiyama[1] and Osamu Koga

Department of Animal Science, Kyushu University, Hakozaki, Fukuoka 812, and [1]Department of Animal Science, Kyushu Tokai University, Kumamoto 869-14, Japan

ABSTRACT—*In vitro* viability and fertilizing capacity of guinea fowl spermatozoa were examined by the method of storing semen at 0°C (in ice). Freshly ejaculated undiluted guinea fowl spermatozoa survived for only three days, in clear contrast to those which were diluted with a phosphate buffer and retained their motility for up to ten days. The number of abnormal spermatozoa increased considerably during the period of preservation of undiluted semen. A relatively high fertility was obtained from both undiluted and diluted semen when they were inseminated shortly after collection and dilution. Duration of fertility of spermatozoa declined from 15 days to 11 days owing to the dilution of semen with the phosphate buffer. The fertilizing capacity of undiluted stored spermatozoa was severely impaired, and their duration of fertility was less than half of that of the diluted unstored ones. The damaged fertility and reduced duration of fertility of stored spermatozoa are likely to be associated with the increased number of deformed spermatozoa during storage.

INTRODUCTION

Male guinea fowl (*Numida meleagris*) have been reported to eject only a negligible amount of accessory reproductive fluid during ejaculation [1], though they possess a special tissue in the ventral cloaca [2] similar to the vascular tissue in roosters [3], the EGR (ejaculatory groove region) in drakes [4] and the TVP (tissue at the vicinity of the papilla of the ductus deferens) in male turkeys [5].

The above-mentioned male birds with the exception of guinea fowl, produce a lymph-like fluid in the cloacal region which is ejected concomitantly with semen during natural copulation and manual semen collection [4, 6–12]. The lymph-like fulid is sometimes called the accessory reproductive fluid in male birds. Roosters and male turkeys eject a tiny quantity of frothy fluid (foam) in addition to a considerable volume of the lymph-like fluid at the time of semen collection [12, 13]. Male quail have foam glands in the cloaca which produce a foam that is ejected with semen at ejaculation [14–17]. In the male pigeon, on the other hand, little or no lymph-like fluid is added to the ejaculated semen (Fujihara, unpubl. data). These findings suggest that the chemical properties of ejaculated semen of male birds may be modified, depending upon the presence or absence of the fluid which is added to semen during copulation.

On the one hand, the seminal plasma amino acids and protein profiles of the guinea fowl have been shown to be similar to those reported for the chicken and turkey [18]. This result might be obtained from the analysis of cock and turkey semen which were collected without addition of the accessory reproductive fluid, suggesting the possibility that these semen samples might be identical in their components to the ductus deferens semen. In the previous report, however, we have confirmed that ejaculated guinea fowl semen were comparable in semen volume and sperm density to the ductus deferens semen [1]. These results lead us to infer that guinea fowl semen may be different in its quality from that of other

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domestic poultry. The present study was undertaken in an attempt to see if guinea fowl spermatozoa have special features peculiar to this species, by examining for *in vitro* viability and fertilizing capacity of spermatozoa.

MATERIALS AND METHODS

Animals

Mature male and female guinea fowl were housed individually in wire cages similar to those used for the rearing of chicken hens, given food and water *ad libitum,* and subjected to a daily photoperiod of 14 hr light/10 hr dark. The diet used here were consisted of the same commercial feed as for laying hens. Egg production was recorded on a daily basis. Male birds were handled for several days prior to the experiment to accustom them to ejaculation by the manual massage method.

Semen samples

The lumbo-sacral massage method available for roosters as a one-man technique [19] was applied to obtain semen from male guinea fowl because we failed to induce male birds to copulate naturally. As guinea fowl semen were very small in volume, the operator aspirated the semen as it appeared at the tip of the copulatory appendage. Semen samples were collected from at least 6 males. Artificially collected semen were pooled immediately after collection and divided equally into two aliquots, one for the undiluted semen group and the other for semen diluted with a phosphate buffer [20] at a semen:extender ratio of 1:3. Sampled semen were placed in test tubes and covered with a piece of aluminum foil.

One part of each aliquot was used to inseminate females immediately after preparation, and the other part was stored in a thermos jar containing ice (0°C) for a given period of time.

Survivability of spermatozoa in vitro

To assess motility and viability of spermatozoa, an aliquot of sperm suspension stored at 0°C (in ice) was taken out at 24 hr intervals and the remainder of each sperm suspension was preserved for further determination of motility and survival time of spermatozoa. Motility of stored spermatozoa was examined by the method of hanging-drop after warming an aliquot of semen to room temperature (24–27°C). The method used for sperm motility measurement was a microscopic visual score of 0 to 5, with 5 representing the highest motility [21]. Measurement of motility at regular intervals was continued until all spermatozoa halted their motile activity.

Morphology of spermatozoa

Morphology and percent abnormality of spermatozoa were determined at a given interval by the staining method described below. One drop of sperm suspension was spread over a slide glass with a coverslip. Spermatozoa smeared over the slide glass were immediately fixed for 1 to 3 hr in a vapor of formalin and then air-dried, after which spermatozoa were stained with carvol-fuchsin solution. Spermatozoan morphology was observed with a light microscope and photographed. Percent abnormality of spermatozoa was calculated by the number of spermatozoa over 1,000 sperm cells on the stained slide glass. Types of malformed spermatozoa were categorized according to the previous reports [10, 22, 23].

For scanning electron microscopy, one aliquot of sperm suspension stored in ice was taken out and washed twice with the phosphate buffer [20] then resuspended in the same buffer solution. A small quantity of sampled semen was dropped on a thin coverslip, spread over, fixed in a vapor of formalin as mentioned above, then air-dried at room temperature. Spermatozoa on the coverslip were further fixed in 2.5% glutaraldehyde in a phosphate buffer [24] for 2 hr at 4°C. The coverslip with spermatozoa was then washed twice with the phosphate buffer and dehydrated, using a graded ethyl alcohol series. The specimens were subsequently critical point-dried and then sputter-coated with gold palladium. Scanning electron micrographs were obtained using a Hitachi SSM-2 microscope operating at 20 Kv.

Fertilizing power of spermatozoa

Aliquots of freshly ejaculated semen, semen diluted 1:3 with the phosphate buffer and semen

stored at 0°C were used for examination of the fertilizing capacity of spermatozoa. Each type of semen sample was inseminated intravaginally, applying the same basic technique as used for chickens, with minor modifications. Briefly, pressure on the abdomen is applied by holding the female between the hands, the oviduct is everted with one hand and insemination carried out with the other. A tuberculine syringe (1.0 ml) calibrated to 0.01 ml divisions, connected with a piece of rubber tubing attached to the end of a syringe with a small glass semen holder (cannula) inserted into the vagina, has been used with success. The semen was most successfully deposited in the vagina at a depth of about 3 to 5 cm. The number of spermatozoa inseminated was adjusted to be equal to the various semen groups by controlling the volume of inseminated semen. Eggs were saved daily for a given period, beginning the second day following single insemination, and incubated weekly in a similar fashion to the method used for hen's eggs, followed by candling after 5 days of incubation. Eggs thought to be infertile were broken open and examined macroscopically for evidence of embryonic development. Duration of the fertility of spermatozoa was determined by mean values of the period (days) in which fertile eggs were produced.

Statistical analysis

The reproduction results, after transformation to arcsine values, were statistically analysed using analysis of variance. If these analyses for variance revealed significance, Student's t-test at P:0.05 was given [25].

RESULTS

In vitro survival time of spermatozoa

The results shown in Figure 1 reveal that freshly ejaculated undiluted spermatozoa of guinea fowl survived for three days, while those diluted with the phosphate buffer retained their motility for ten days. A definite difference between the two semen groups was also found in sperm motility. Spermatozoa suspended in the buffer solution maintained considerably higher motile activity through their

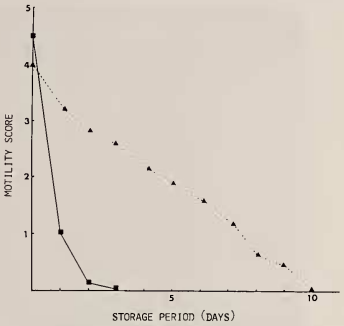

Fig. 1. Viability of guinea fowl spermatozoa preserved at 0°C.
■—■ Undiluted semen. ▲—▲ Semen diluted 1:3 with phosphate buffer.

survival, in clear contrast to the undiluted semen in which the motility of spermatozoa declined rapidly after one day of preservation at 0°C (in ice) (Fig. 1). The difference in sperm motility and survival time between undiluted and diluted semen samples was statistically significant ($P<0.05$).

In the course of preservation of sperm suspension in ice, the number of abnormal spermatozoa in undiluted semen increased markedly: more than 80 percent of the spermatozoa were deformed at the end of the storage period. Percent abnormality of stored spermatozoa increased along with the prolonged preservation period: even in diluted semen, a considerable number of abnormal spermatozoa was observed towards the end of the preservation period.

Figure 2 shows the rate of deformed spermatozoa on the first day of preservation, being 70% in the undiluted semen group and 40% in the semen diluted (1:3) with the optimal diluter. The obvious difference between the two semen groups was statistically significant ($P<0.05$). Most of the deformed spermatozoa observed at the beginning of storage were crooked-necked [23] or neck-bent [10] spermatozoa (Fig. 3). The deformed spermatozoa with crooked or bent necks were characterized by the fact that their heads and tails were so closely bent to each other on the middle-piece that they appeared as almost straight lines, just like

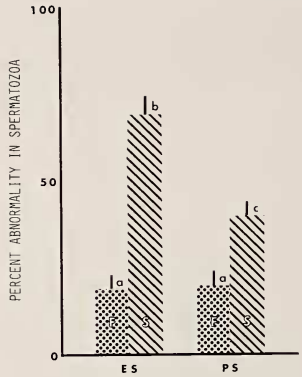

Fig. 2. Percentage of abnormal spermatozoa in fresh and stored guinea fowl semen.
ES, ejaculated semen; PS, semen diluted 1:3 with phosphate buffer. F, fresh semen; S, semen stored for 24 hr at 0°C. Values with different letters are significantly different ($P<0.05$).

normal spermatozoa. The crooked-necked spermatozoa showed forward movement, just as normal ones did, and their motility and viability were the same. The percentage of abnormal spermatozoa in semen increased with the lapse of storage time and its incidence was greater when semen was undiluted and stored than when it was diluted with the phosphate buffer (Fig. 2). Malformed spermatozoa other than the neck-bent ones, i.e. detached head, deformed mid-piece, abnormal tail [22] also increased in number with prolonging storage period. Another peculiarity to guinea fowl spermatozoa was that a small number of spermatozoa possessed swollen heads (Fig. 4). The general morphology of normal guinea fowl spermatozoa was quite similar to that of other domestic birds (Fig. 5).

Fertilizing capacity of spermatozoa

Table 1 shows that a relatively high fertility was obtained from both undiluted and diluted semen when inseminated shortly after collection and treatment, though there was no significant difference ($P>0.05$) between the two semen types. One major difference between undiluted and diluted semen groups was seen in the duration of fertility, which was 15.2 days for undiluted semen and 10.9 days for diluted semen (Table 1). A statistical difference ($P<0.05$) was found between the two semen samples.

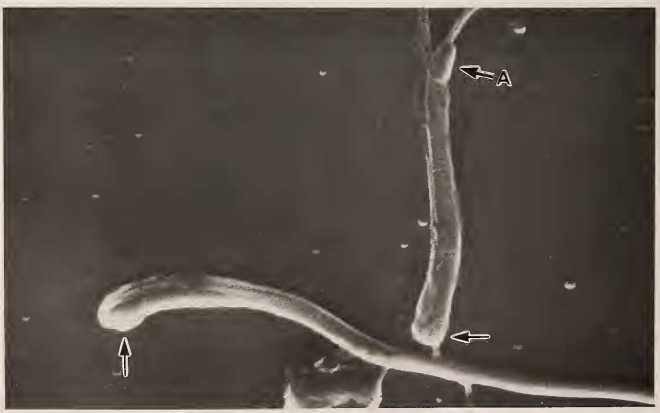

Fig. 3. Deformed guinea fowl spermatozoa with crooked neck (arrows). Note that the head and tail were so closely bent to each other upon middle-piece. A, acrosome. ×4750.

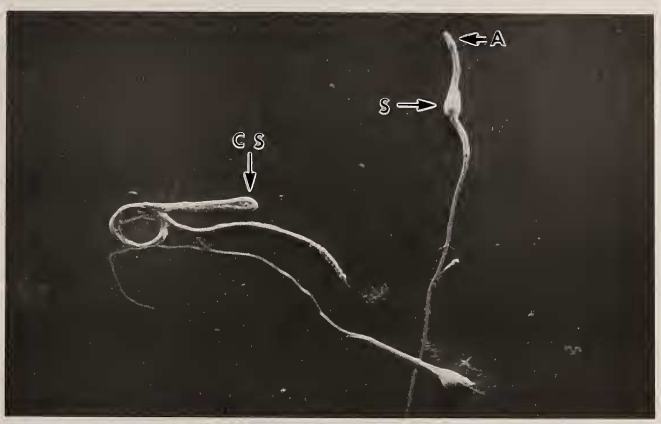

FIG. 4. Abnormal guinea fowl spermatozoa with crooked neck (CS), and swollen head (S). A, acrosome. ×1690.

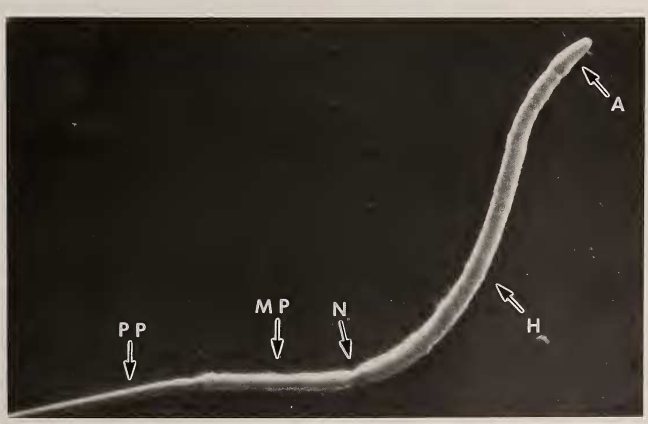

FIG. 5. Scanning electron micrograph showing morphologically normal guinea fowl spermatozoon. A, acrosome; H, head; MP, middle-piece; N, neck; PP, principal piece. ×5890.

Compared to freshly ejaculated spermatozoa, the fertilizing capacity of undiluted and stored spermatozoa was severely impaired (Table 1). Even for semen extended with optimal buffer solution, the percentage of fertility was lower when semen samples were preserved at a low temperature (0°C). Duration of fertility of diluted preserved spermatozoa (5.7 days) was half that of diluted unstored ones (10.9 days) (Table 1). Most of the females which received undiluted stored

Table 1. Percentage of fertile eggs after single insemination with fresh and stored semen of guinea fowl

Semen	No. of females inseminated	No. of sperm inseminated	Percent fertile	Duration of fertility
		($\times 10^6$)	(%)	(Days)
Fresh				
Undiluted	6	68	68.3[a]	15.2[a]
Diluted*	8	64	66.8[a]	10.9[b]
Stored**				
Undiluted	6	62	3.7[b]	1.2[b]
Diluted*	6	62	44.8[a]	5.7[a]

*Semen were diluted with a phosphate buffer (1:3).
**Semen were stored for 24 hr in a thermos jar containing ice.
[a,b]Values within each column with different superscripts are significantly different ($P<0.05$).

semen produced no fertile eggs after single insemination, thus resulting in very short duration of fertility on average. Semen diluted with the phosphate buffer and stored had considerably higher fertility (44.8%) than undiluted stored semen (3.7%), but duration of fertility (5.7 days) was reduced noticeably in comparison with unstored semen groups (10.9 days).

DISCUSSION

In vitro viability of undiluted guinea fowl spermatozoa was maintained for only three days, in clear contrast to those which were diluted with the phosphate buffer and retained their motility for up to ten days (Fig. 1). This suggested that freshly ejaculated undiluted guinea fowl spermatozoa were unable to withstand *in vitro* storage for a long period of time in comparison with spermatozoa from roosters, drakes and male turkeys in which spermatozoa survived for a considerable period of time even when their semen were not diluted with semen extender [26–28]. Judging from sperm motility and viability, guinea fowl spermatozoa seem to be quite different from those from other domestic birds. It is not known what factors are responsible for the shorter life span of undiluted spermatozoa of guinea fowl during storage.

As reported previously [1], only a negligible amount of the lymph-like fluid was added to guinea fowl semen at the time of ejaculation, indicating that ejaculted guinea fowl semen is more or less comparable in its component to the ductus deferens semen. This means that the ductal spermatozoa of guinea fowl is strikingly poor in storability *in vitro*. Undiluted ductus deferens spermatozoa from male turkeys and drakes remained motile *in vitro* for a period ranging from 10 to 15 days, a period similar to those diluted with the accessory reproductive fluid and the phosphate buffer [27, 28].

Spermatozoa from the ductus deferens of pigeon and quail could not withstand *in vitro* storage when they were not diluted (Fujihara, unpubl. observation). These results indicate that spermatozoa from guinea fowl, quail and pigeon have some characteristics in their function which differ from spermatozoa from other domestic birds. The vas deferens fluid is considered in general to be an isotonic, physiological medium for spermatozoa, but if the frequency of ejaculation is not optimal, the chemical environment in the vas deferens is not likely to be ideal for the survival of spermatozoa [29]. The difference in life span of spermatozoa between guinea fowl (in this study) and other male birds may be attributed to the natural characteristics inherent in spermatozoa from the respective species of birds.

As for fertilizing capacity of spermatozoa, a few studies have centered on fertilizability of guinea fowl spermatozoa. The fertility (68.3%) of freshly ejaculated undiluted guinea fowl spermatozoa reported here was considerably lower than that (90%) reported previously [30]. This value was

also small in comparison with the fertility of spermatozoa from roosters, turkeys and drakes which have so far been reported by investigators. The lowered fertility of guinea fowl sperm in this study may be partly due to the small number of sperm inseminated. On the contrary, the duration of fertility (15.2 days) of guinea fowl spermatozoa described here was a little longer than the value reported by Belshaw [31] who noted seven days as the fertile period for guinea fowl spermatozoa.

On the other hand, undiluted stored guinea spermatozoa lost its fertilizing capacity after two days of preservation. Even semen extended with optimal buffer solution showed impaired fertility following preservation for 24 hr at a low temperature despite the fact that spermatozoa could remain motile for as long as ten days.

In the past, many attempts have been made to find diluent media, the ideal temperature for preservation and other physical conditions for the prolonged storage of avian semen. The best fertility results were always obtained when diluted semen was used within a very short time after collection. The reason for the rapid reduction in fertilizing ability of preserved avian sperm has yet to be clarified. For reviews of poultry semen preservation and factors affecting fertility with artificial insemination, the reader should refer to Cooper [32, 33], Lake [34–36] and Sexton [37].

Even in mammalian spermatozoa stored at a low temperature, fertility correlated very poorly with motility. Low temperature storage of liquid semen could yield spermatozoa of apparently good motility and percentage motile, but of low fertility [38].

From the morphological point of view, increased numbers of crooked-necked or bent-neck spermatozoa were observed in stored guinea fowl semen, as is also the case in semen from cocks [10, 23], drakes [27] and male turkeys [28]. Guinea fowl spermatozoa with bent-neck were still strongly motile, just like normal spermatozoa were, but incapable of fertilizing eggs. Higher incidence of deformed spermatozoa in guinea fowl semen stored *in vitro* was thought to be associated with lowered fertility. A similar phenomenon to this has been demonstrated in semen from drakes [27] and male turkeys [28]. Significant ($P<0.01$) correlation has also been observed between crooked-necked spermatozoa and fertility in chickens and turkeys [23, 39]. It is, thus, considered that in avian spermatozoa, motility score is a poor indicator of fertility, and that the number of abnormal spermatozoa may give a better indication of fertility than estimation of visual motility.

The reports on the fluid contained in ejaculated avian semen suggest that the lymph-like fluid and frothy fluid (foam) originated in the cloacal region of male birds may not always be indispensable for semen and spermatozoa. Based on these findings, guinea fowl and pigeon, which do not produce fluids in the cloacal region, may be considered to be unique in the production of semen and sperm physiology.

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